Antibodies and protection in systemic *Salmonella* infections: do we still have more questions than answers?

Pietro Mastroeni[1*] & Omar Rossi[2]

1: University of Cambridge, Department of Veterinary Medicine, Madingley Road CB3 0ES, Cambridge (UK)

2: GSK Vaccines institute for Global Health, Via Fiorentina 1, 53100, Siena (Italy)

*** Corresponding author:** Dr Pietro Mastroeni, University of Cambridge, Department of Veterinary Medicine, Madingley Road CB3 0ES, Cambridge (UK), e-mail: pm274@cam.ac.uk

Keywords: Antibodies, IgM, IgA, IgG, Systemic infections, *Salmonella*, vaccines

Abstract

Salmonella causes grave systemic infections in humans and other animals and provides a paradigm for other diseases where the bacteria have both intracellular and extracellular lifestyles.

New generations of vaccines rely on the essential contribution of the antibody responses for their protection. The quality, antigen specificity and functions associated with antibody responses to this pathogen have been elusive for a long time. Recent approaches that combine studies in humans and genetically manipulated experimental models, and exploit awareness of the location and within-host life cycle of the pathogen, are shedding light on how humoral immunity to *Salmonella* operates. However, this area of research remains full of controversy and discrepancies.

The overall scenario indicates that antibodies are essential for resistance against systemic *Salmonella* infections and can express the highest protective function when operating in conjunction with cell-mediated immunity. Antigen specificity, isotype profile, Fc-gamma receptor usage and complement activation are all intertwined factors that still arcanely influence antibody-mediated protection to *Salmonella*.

Introduction

Several serovars of *Salmonella enterica* cause systemic diseases in humans and other animals. The global estimated burden of typhoid fever (serovar Typhi) is over 21M illnesses and 200,000 deaths with sustained high incidence in Southeast Asia and endemic/epidemic occurrence increasingly reported in Africa (1-6). Paratyphoid fever (serovars Paratyphi A, B and C) has an estimated 5.4M illnesses worldwide (3). Invasive non-typhoidal *Salmonella* (iNTS) serovars (*e.g.* Typhimuriun and Enteritidis) are a leading cause of lethal sepsis and severe relapsing infections in young children and immune-compromised individuals, especially in countries of the sub-Saharan African region (6-12) with an estimated 3.8M illnesses leading to 680,000 deaths annually and very high case-fatality ratios (20%) (7, 11). Antimicrobial resistance (AMR) is an increasing problem in tackling many bacteria including *Salmonella* (7, 11, 13-15).

Why is it important to gain a better understanding of antibody-mediated immunity to *Salmonella*?

There is extensive international consensus on the urgent need for better and affordable vaccines against systemic *Salmonella* infections. Vaccination has the potential for a high economic and health impact in fighting AMR infections (16-19). Several classes of vaccines against systemic *Salmonella* disease have been considered in the past decades (20, 21). These vary in their ability to induce protective cell-mediated and humoral immunity with, broadly speaking, live attenuated vaccines being more efficient than non-living preparations at eliciting Th1 type T-cell immunity known to contribute to host resistance to this bacterium (22, 23).

Despite the superior protective activity shown in animal models, live *Salmonella* vaccines can cause lethal infections in immune-compromised hosts (24-27). Mutants of *Salmonella*, including those that have been considered so far as vaccine candidates, retain virulence and can rapidly kill immune-suppressed mice

(24-27). Mice that lack T-cell functions (25, 27) and mice co-infected with malaria are very susceptible to infection with live *Salmonella* including mutants that have been considered as vaccine candidates (28, 29). Efforts to identify single gene mutations for the development of live vaccine candidates that would be completely safe (totally unable to grow to high numbers) in severely immune-deficient animals have been unsuccessful (24). This raises some concerns for the use of live vaccines in endemic areas with a higher incidence of immune-suppressive conditions. For example, HIV and malaria are co-morbidities that make humans more susceptible to systemic *Salmonella* infection leading to severe, often fatal disease, and therefore pose dangers to the use of live vaccines (30, 31). These co-morbidities are widespread and epidemiologically co-localize with areas of the developing world where there is a high incidence of epidemic or endemic systemic salmonelloses and therefore where anti-*Salmonella* vaccines are most needed (9, 20, 31, 32).

Mainly for safety reasons, non-living vaccines are currently being considered as prime candidates for immunization against *Salmonella* diseases. The protective ability of these vaccines relies largely on the induction of antibodies. If we are to use non-living vaccines as tools against systemic salmonelloses, it is therefore essential to rationally optimize the antibody responses induced by these preparations. This knowledge would also be immensely useful to understand how those comorbidities that impair antibody-mediated functions increase susceptibility to disease and to design vaccine strategies that can at least in part reverse these immune suppressing conditions. This will need to be based on a clearer understanding of the qualitative and functional features of the protective antibody response against *Salmonella*.

This article will briefly outline the factors that influence the protective efficacy of the antibody response to systemic *Salmonella* infections and will embed antibody functions in the context of the location and spread of the bacteria during the infection process. The mini-review will also highlight the interactions

and dual requirement of T and B-cell mediated immunity both in the engenderment of antibody and T-cell responses and in the expression of *in vivo* resistance to the pathogen.

How can antibodies protect against a pathogen that has an intracellular lifestyle?

The relative importance of antibodies and cellular immunity in host resistance to systemic *Salmonella* infections has been a matter of debate for a long time.

The controversy initially originated from uncertainties in the location of the bacteria within the tissues of an infected host. It is undisputable that in many animal species, including humans, *Salmonella* resides inside phagocytes and grows within these cells using an armoury of genes and effectors to foster its replication and evade intracellular killing (24, 33-40). Evidence from the mouse model on the role of the phagocyte innate resistance gene *Ity* (now known as *Scla11a1*) in the control of bacterial division *in vitro* and *in vivo* as well as studies where the bacteria could be seen to grow within cultured phagocytic cells corroborated these views (41-44). However, evidence for extracellular growth and lack of intracellular replication had also been produced, albeit based on electron microscopy studies where infections were allowed to progress to very high, *pre-mortem*, bacterial numbers in the tissues. In these studies intracellular bacteria were seen to undergo various stages of degradation inside macrophages and granulocytes and therefore it was inferred that *Salmonella* was more likely to be an extracellular pathogen and its virulence directly related to its antiphagocytic property (45).

The debate was further fuelled by immunological studies in both experimental animals and humans. On the one hand evidence was provided for the protective role of antibodies being limited to the very early stages of parenteral experimental infections, when the bacteria have not yet reached an intracellular location, with no detectable effect on their later growth in the tissues (46, 47); on the other hand the

protective effect of whole-cell non-living typhoid vaccines, that induce mainly humoral immunity, and Vi vaccines that do not directly contain *Salmonella*-specific T-cell antigens was undeniable (48-53). However, protection by passive transfer of antibodies or T-cells alone and by non-living vaccines was seen only in host-pathogen interactions where the infection is naturally mild and sublethal, such as challenging resistant mice with either virulent or weakly virulent strains or susceptible mice with weakly virulent strains (46, 50).

These discrepant views on the relative importance of humoral and cellular immunity to *Salmonella* were largely reconciled by studies showing that passive transfer of both antibodies and T-cells, but neither alone, could protect recipient innately susceptible animals against fatal systemic *Salmonella* disease (54). More recently the essential role of both antibodies and T-cells in host resistance to *Salmonella* has been corroborated by additional evidence obtained from human studies. For example, clinical observations indicate that lack of antibodies at young age correlates with the incidence of iNTS in African children (55, 56), despite these children acquire *Salmonella*-specific CD4 Th1 cell immunity very early in life; only when antibodies are developed in addition to T-cell immunity full protection is achieved (57). This resistance is then abrogated by comorbidities that can affect either cellular or antibody-mediated functions (29, 58-62). For example, HIV that has a profound effect of $CD4^+$ T-cell immunity and malaria that impairs phagocyte functions increase susceptibility to systemic *Salmonella* infections such as iNTS. A similar increase in susceptibility is seen in patients with deficiencies in cytokines such as IFNγ and IL12 and in their receptors leading to lack of activation of phagocytes and likely also T-cell immunity (9, 27, 63-67).

Salmonella: a bacterium with a complex intra and extracellular pathogenesis. Where do antibodies target _Salmonella_?

Salmonella infections are normally acquired by the oral route and reach the blood after invading the gastrointestinal tract (68). In the blood, the bacteria can be found either as extracellular bacteria or associated with $CD18^+$ cells (69) (Figure 1, A). Extracellular *Salmonella* in the blood can be targeted by antibodies that enhance their uptake and killing by resident phagocytes of the spleen and liver (46, 70, 71) and can potentially lyse them *via* activation of the complement classical pathway (56). From the blood, *Salmonella* reach an intracellular location within phagocytes of the liver, spleen and bone marrow (70, 72, 73) (Figure 1, B). Early in infection *Salmonella* reaches mainly splenic red pulp ($F4/80^+$, MSR-A^{low}) and marginal zone macrophages (MSR-A^+) (74). In the liver, *Salmonella* localizes preferentially in the resident Kupffer cells. During the infection bacteria can also be found in dendritic cells or B-cells (36, 74-77). As the infection progresses and bacteria reside and possibly grow intracellularly (Figure 1, C), inflammatory cells infiltrate the initial unicellular foci of infection, to form multicellular pathological lesions surrounded by normal tissue (Figure 1, D). These lesions contain polymorphonuclear phagocytes (PMNs) during the first few days of infection, later replaced by inflammatory macrophages (36, 78).

Once *Salmonella* have homed inside host cells, their intracellular location would render the bacteria inaccessible to antibodies, making it difficult to envisage a role of humoral immunity in protection. Detailed analysis of intracellular bacterial densities over the course of experimental infections in mice have shed light on how and where the bacteria can become vulnerable to antibodies. In fact, microscopic observation of immune-labelled and/or fluorochome-expressing intracellular bacteria in the spleen and liver revealed that the majority of infected phagocytes contain very low intracellular numbers at any time of the infection, irrespective of overall the net growth of the bacteria in the organs (77). The infection

process is underpinned both by intracellular growth/survival and by an increase in the number of infected cells and multicellular pathological lesions and not by increases in the number of visible intracellular bacterial per phagocytes (24, 77, 79) (Figure 1, D). This is due to the continuous re-distribution of the bacteria from infected host cells to uninfected ones, and in the spread of individual microorganisms to new infection foci (Figure 1, E), mediated by the *Salmonella* Type three secretion system (T3SS) encoded by the *Salmonella* Pathogenicity Island 2 (SPI-2) (80, 81). *Salmonella* infections are therefore dispersive processes where bacteria have an intracellular phase of growth and an extracellular one of spread.

Is the dispersiveness of the infection a plausible explanation for the role of antibodies in protection against *Salmonella*? It would be reasonable to postulate that in a dispersive infection process, cell-mediated immunity enhances the antimicrobial functions of phagocytes, therefore affecting the fate of the intracellular bacteria (82-86); antibodies would opsonise the extracellular bacteria in transit between cells and target them to activating cellular receptors, thus increasing the antimicrobial activity of otherwise naive phagocytes at new infection foci (87, 88). Antibodies would therefore be expected to have a major impact on the process.

However, we know from a large body of data that antibodies alone, in the absence of cell-mediated immunity, are unable to modify the net growth rate of *Salmonella* in the spleen and liver of an infected experimental animal (21, 46, 50). For example, adoptive transfer of *Salmonella* specific immunoglobulins or immunisation with non-living vaccines surprisingly does not affect the growth curve of the bacteria *in vivo*. An effect of antibodies is visible only in the first few hours of the infection where the initial kill of the inoculum is enhanced (46). This early protective effect of antibodies is more evident when experimental animals are challenged *via* the intraperitoneal route, where antibodies most likely

160 accelerate capture of the bacteria by peritoneal phagocytes and therefore abort extracellular growth in
161 the peritoneal cavity. The use of molecularly tagged and therefore individually traceable, *Salmonella*
162 populations, combined with mathematical modelling and statistical analysis of data has further confirmed
163 the inability of antibody to significantly affect the growth and spread of bacteria in the body. This research
164 approach has confirmed that, in the early stages of the infection, bacteria spread from cell to cell within
165 each organ, later followed by systemic spread of bacterial populations between distant body sites
166 coinciding with the appearance of bacteria in the blood (89). Using this system, it became clear that only
167 live vaccines (which induce both humoral and cell-mediate immunity) would be able to control
168 bacteraemia and restrain growth and spread of the bacteria in the body. Conversely, mice where
169 antibodies were the only vaccine-mediated effector mechanisms (i.e. mice immunised with killed vaccines
170 or depleted of T-cells after immunisation) would not be able to control the spread of the bacteria or abort
171 bacteraemia (71).
172
173 **Antigen specificity and protection.** *Salmonella* infections induce antibody responses against a large array
174 of surface, periplasmic and cytoplasmic antigens in humans and other infected animals. Immuno-reactive
175 antigens have been detected using ELISA, immunoblotting and protein microarrays (90-94). These include
176 lipopolysaccharide (LPS) antigens, porins, lipoproteins, fimbriae, flagella, heat shock proteins and in some
177 serovars, the Vi surface polysaccharide antigen (95, 96). Several antigens have been identified as targets
178 of the protective antibody response. The O-antigen, consisting of sugar repeats exposed on the bacterial
179 surface, is a prime target of protective antibodies with some epitopes being more protective than others.
180 Immunodominant serovar-specific O-antigens (e.g. O:4, O:9) determine to a large extent the specificity of
181 protection between serovars and induce antibodies that are more protective than the ones directed

against O-antigens shared among different *Salmonella* serogroups (e.g. O:12) (97-100). The Vi antigen is immunogenic and was shown to confer protective immunity in human volunteers and in field trials, both as a native polysaccharide and as a protein conjugate (49, 51, 53, 101-103).

The functional role of the antibody response to porins is still not fully clarified. Antibody responses against some porins, but not others, are protective in animal models (104, 105). For example, antibodies to the trimeric OmpD porin, but not the monomeric and abundant OmpA protein, protect mice against parenteral challenge. Furthermore, OmpD is a candidate antigen for iNTS vaccines (20, 105, 106). The reasons for the different protective ability of OmpA and OmpD have been elegantly postulated to be due to different accessibility of antibodies to OmpD at the bacterial surface. Both OmpA and OmpD are located in the outer membrane of *Salmonella* in a position potentially shielded by the LPS-O side chain. However, OmpD creates a larger footprint than OmpA in the O-antigen layer, sufficient for a single IgG can gain access to the most-exposed surface loop epitopes of OmpD (105). It remains unclear how antibodies to OmpD mediate SBA given that at least two Ig need to come together for activation of the classical pathway of the complement. The conclusion that OmpA is poorly accessible by IgG also clashed with data showing its binding by human recombinant IgG specific for a mimotope inserted in OmpA (107).

Antibodies to flagellin are detectable in infected individuals and targeting this antigen can mediate opsono-phagocytosis (87, 93).

Which is the main protective mechanism of anti-*Salmonella* antibodies?

Following their binding to the bacterial surface, antibodies can exert antibacterial efficacy mainly in two ways. Either by increasing phagocytosis of bacteria *via* targeting the bacteria to specific receptors on the surface of immune cells; or by direct bactericidal activity mediated by the activation of the classical

complement pathway (serum bactericidal activity, SBA). Both these mechanisms are likely to operate in *Salmonella* infections. Early studies showed that clearance of *Salmonella* from the blood of mice is dramatically accelerated by immune serum suggesting a role for antibodies in the enhancement of phagocytosis (70). More recently, evidence for an enhancement of opsono-phagocytosis by *Salmonella* antibodies has been corroborated by *in vitro* systems. Sera from humans vaccinated with live attenuated *S.* Typhi enhance opsono-phagocytosis *in vitro* with IgG playing a major role(108). Engagement of opsonised bacteria with the activating Fc-gamma receptors, on the surface of murine or human phagocytes results in increased uptake of the bacteria, increased production of reactive oxygen intermediates (ROI) and enhanced antibacterial functions of the infected cells (87, 88, 107). Opsonization drives increases in both the number of phagocytes that ingest bacteria and in the efficiency of each individual cell to ingest *Salmonella,* as indicated by the higher intracellular numbers per phagocyte of opsonized *versus* non-opsonized bacteria (88, 107). Antibodies have also shown to be essential for optimal phagocytosis of iNTS strains by peripheral blood cells from Africans (55).

Antibody-dependent, complement-mediated SBA correlates with susceptibility to iNTS in African children (56) and therefore SBA has been often taken as an indication of the functional activity of antibodies when testing new vaccines against iNTS (109). However, the role of SBA as a true mechanism of antibody-mediated protection must be evaluated with caution. Studies that used human blood and sera to compare the kinetics of SBA and phagocytosis of iNTS clinical isolates, show that phagocytosis allows bacteria to escape the blood and establish intracellular infection before they are killed by the complement membrane attack complex (110). This would indicate that opsono-phagocytosis is likely to be the prevailing antimicrobial mechanism mediated by anti-*Salmonella* antibodies. Furthermore, no correlation was found

between protection afforded by live vaccination and SBA in a controlled human typhoid challenge model(111).

Complement or Fc-gamma receptors?

The complement system and Fc-gamma receptors (Fc*γ*Rs) can potentially play a crucial role in antibody-mediated immunity against *Salmonella* diseases. However, their relative importance is unclear.

Complement-mediated SBA can result in bacterial killing, but, as discussed above, it is unclear whether this mechanism is relevant for immunity to *Salmonella*. Complement appears to be essential for antibody dependent-opsonophagocytosis of iNTS strains by human blood phagocytes and for the production of reactive oxygen intermediates and bacterial killing (55). However, binding of antibody-opsonized *Salmonella* to FcγR on human and murine cells in the absence of complement also enhances phagocytosis and ROI-mediated bacterial killing, with the activating FcγRI playing a major role (88, 107).

In vivo studies also provide evidence for a role for both complement and FcγR, their relevance depending on the experimental model used. When mice lacking Fc*γ*RI, II, III and IV (Fc*γ*R KO), or mice lacking the complement C3 component (C3 KO), or all four Fc*γ*R and C3 (Fc*γ*R/C3 KO), were passively immunized with anti-LPS O4 IgG2a monoclonal antibodies and subsequently infected parenterally with *Salmonella* Typhimurium, only Fc*γ*R KO animals showed a significant reduction in the bacterial loads in the liver, spleen and mesenteric lymph nodes (112), at a level similar to the ones observed in wild-type control animals. In this model therefore, the role of complement prevails on that of Fc*γ*Rs. However, when mice lacking Fc*γ*RI, II, III and mice lacking C3 were immunised with a live attenuated vaccine and later challenged orally with virulent *Salmonella*, only the Fc*γ*R-deficient mice succumbed to the infection (113) indicating a prevailing role of Fc*γ*R.

247 In summary, evidence for a role of both FcγR and complement has been provided, but firm conclusions
248 on their relative importance are difficult to draw due to discrepancies between experimental conditions,
249 models and host species.
250
251 **Does quality of the antibody response matter?**
252 The qualitative traits of the antibody response have a great effect on its function and potency. The isotype
253 profile has effects on the binding of antibodies to FcγR receptors and on efficiency of complement
254 activation. This in turn has effects on SBA, opsono-phagocytosis and on the enhancement of the
255 intracellular antibacterial mechanisms of phagocytes.
256 Virtually all classes of Ig are produced in response to infection with *Salmonella*. As expected, IgM appear
257 early after infection and usually followed by IgG and IgA (92, 95, 114-117). Different isotype profiles are
258 seen following natural infection or vaccination with live or non-living vaccines (22, 118). Some non-living
259 vaccines can induce isotype-switched responses to *Salmonella* polysaccharide antigens indicating that the
260 T-cell responses induced by these preparations may not be able to mediate the suppression of bacterial
261 growth in the tissues, but are sufficient to support isotype switching of the Ig response. For example, IgG
262 responses are detected following vaccination with Vi-conjugate vaccines (51, 119, 120); interestingly
263 outer membrane vesicle vaccines (OMV) are capable of inducing highly effective IgG2a and IgG2b in mice
264 (109); live vaccines induce high titers of all IgG subclasses including higher levels of IgG2a (22).
265 The isotype profile of the antibody response impacts on its protective function. The efficacy of individual
266 subclasses has been studied in murine and human systems and this area of research is not devoid of
267 controversy. In murine studies, polyclonal and monoclonal IgM as well as IgA to *Salmonella* polysaccharide
268 antigens, were found to be highly protective and in some cases more protective than IgG (100, 121, 122).

Conversely, opsonization of *Salmonella* with O4-specific IgA, IgG1, IgG2a, or IgG2b, but not IgM monoclonal antibodies, resulted in cell-dependent bacterial killing *in vitro*. In *in vivo* passive immunization studies, IgG2a and IgG2b O4-specific monoclonal antibodies provided higher functional activity than IgA, IgM and IgG1 by decreasing the bacterial load in the blood and tissues (123).

A role for IgA in protection against *Salmonella* is shown by the increased susceptibility to infection of polymeric immunoglobulin receptor ($pIgR^{-/-}$) knock-out mice, which are unable to bind and actively transport dimeric IgA to the mucosae (124) and by the protective ability of IgA monoclonal antibodies *in vivo* (125).

The relative potency of individual isotypes can be dependent on the antigen that is targeted. In fact, differently for what seen in the case of antibodies against the LPS-O antigen, it has been shown that mice lacking IgG1, but not lacking IgG2a are substantially less protected after immunization with the OmpD porin than wild-type controls. Immunization with OmpD was maintained in t-bet deficient mice that do not produce IgG2a. This is consistent with IgG1 having an important role for protection after immunization with OmpD, but IgG2a being less important (126).

The relative potency of human IgG was studied in an *in vitro* system where OmpA and flagella were tagged with a foreign CD52 mimotope (TSSPSAD). The bacteria were opsonized with a panel of humanized recombinant CD52 antibodies that share the same antigen-binding V-region, but have constant regions of different subclasses. This work revealed that, although opsonization with all the IgG subclasses increase *Salmonella* uptake by human phagocytes, differences in potency can easily be revealed. IgG3 resulted in the highest level of bacterial uptake and the highest average bacterial load per infected cell, which was closely followed by IgG1, then IgG4 and lastly IgG2. Phagocytosis mediated by IgG1, IgG3 and IgG4 had a higher dependency on FcγRI than FcγRIIA, whereas IgG2-mediated phagocytosis required FcγRIIA more

than FcγRI (87, 107). Therefore, both the subclass of human IgG and the type of FcγR that is available for antibody binding affects the function of anti-*Salmonella* antibodies.

Some IgG subclasses can be detrimental to the antimicrobial function of the antibody response. In fact, the inability of blood from HIV patients to kill *Salmonella* is due to an inherent inhibitory effect of anti-LPS antibodies. This inhibition is dependent on high concentrations of antibodies and strongly associated with IgA and IgG2 anti-LPS antibodies, possibly related to the poor ability of IgA and IgG2 to activate complement, and deposition of complement at sites where it cannot insert in the bacterial membrane (127).

Crosstalk between B-cells and T-cells and quality traits of the antibody response.

T-cell responses can be detected in animals and humans following infection with live *Salmonella*(128-130). Isotype switching and production of anti-protein antibodies requires the presence of T-cells (27, 118); for example, T-cell deficient athymic mice produce mainly IgG3 and IgM antibodies to lipopolysaccharide, whereas euthymic mice can produce IgM, IgG1, IgG2a, IgG2b, and IgG3 anti-LPS and anti-*Salmonella* protein antibodies (27). Similarly, some classes of non-living vaccines only elicit a restricted isotype repertoire (22). The reciprocal interaction between T- and B-cells is essential for the development of both humoral and cell-mediated immunity to *Salmonella* (Figure 2). In the absence of functional B-cells, the onset of T-cell immunity is impaired, albeit not abrogated in mice (131-134). Cytokine production and antigen presentation via MHC Class II molecules from B-cells are essential for activation of Th1 and Th17 responses to *Salmonella,* as shown by studies in bone marrow chimera mice where only B-cells are deficient in selected immunological functions (135). Interestingly B-cells can engender T-cell responses *via* engagement of innate immune receptors early in infection and *via* specific

313 activation of the antigen-specific B-cell receptor later in the disease. These functions instigate a loop
314 where T-cell activation in turn contributes to the isotype profile of the response. In fact, antigen-specific
315 IgG2c primary response are dependent on MyD88 signaling to B cells, while other Ig classes are not (IgG1
316 and IgG3) or much less so (IgG2b, IgA). Lack of MyD88 signaling in B-cells of chimeric mice results in
317 impairment of development of IFNγ effector T cells, a likely contributory factor in the lack of IgG2c (136).
318
319 **Key considerations for the development of *Salmonella* Vaccines.**
320 Antibodies are an essential component of the protective immune response to *Salmonella*. An ideal vaccine
321 would therefore be the one able to induce both antibody responses and protective cellular immunity.
322 The induction of some level of T-cell immunity is essential whichever the choice of vaccine. T-cell
323 immunity induced by natural infection or live vaccines is sufficient to support the isotype switching and
324 affinity maturation of the antibody response, but also to mediate the suppression of the growth of virulent
325 bacteria when either resistant or susceptible animals are re-infected. On the contrary, T-cell immunity
326 induced by some non-living vaccines is sufficient to support isotype switching and anti-protein responses
327 (109, 118), but not protective cellular responses. Why this level or type of T-cell immunity is unable to
328 activate phagocytes and curtail bacterial net growth in the tissues, still remains one of the main
329 unanswered questions in bacterial vaccinology.
330 Antibodies alone can have an impact on the very early stages of the infection, when they enhance
331 bacterial killing before *Salmonella* have reached an intracellular location within phagocytes (46, 71);
332 however they have no effect on the net growth of bacteria in the tissues and surprisingly on the control
333 of bacteraemia (9, 46, 60, 71, 127, 137). Therefore, it is likely that in individuals who do not have specific
334 Th1 memory, vaccine-induced antibody responses protect by preventing the establishment of the

infection following transmission of the pathogen. The situation would be different in populations where background cellular immunity is present. For example, those vaccines that induce mainly antibody responses would be more effective in disease-endemic areas, where a background of cellular immunity is already present in the population, likely due to low grade pre-exposure to the pathogen and/or cross-reactive antigens of other micro-organisms. Interestingly, young African children develop Th1 immunity to *Salmonella* very early in life, but remain susceptible to iNTS until they acquire *Salmonella*-specific antibodies (57); the aim of an effective iNTS vaccine for children would therefore the be induction of antibodies early in life. A lower protective efficacy of Vi vaccines against typhoid fever has been detected in volunteers in a controlled human challenge study compared to field studies, further suggesting the possibility that a different immunological exposure background due to the geographical area may affect vaccine efficacy(102, 103, 138).

The development of both safer live attenuated vaccines and non-living vaccines would be desirable; the former would be more suitable for travelers where elicitation *de novo* of both T- and B-cell immunity is required; the latter vaccines would be suitable for use in endemic areas where, as discussed earlier, increased safety is a prerequisite and induction of antibodies would suffice.

Optimization of the immune response is important especially for non-living vaccines whose efficacy is likely to be entirely based on antibodies. The importance of the isotype profile in relation to antigen specificity and function has been touched upon above with some representative examples.

Greater efforts are needed to optimize antibody responses induced by vaccines to be used in areas where immune suppressive co-morbidities co-localize geographically with the target disease. An example is provided by malaria. This disease has a dual effect on humoral immunity. Firstly, malaria can suppress the acquisition of anti-*Salmonella* antibodies (59, 139). Secondly, malaria can impair complement levels (61)

and suppress the antimicrobial functions of phagocytes (28, 58, 140), therefore potentially not allowing the expression of antibody-mediated resistance (55, 88). More research is needed to identify vaccine solutions that can overcome these problems. In fact, iNTS vaccines for Africa must induce immune responses optimized to confer resistance in the presence of multiple and varying underlying comorbidities.

The optimal choice of antigens is still debatable and several single-antigen vaccines are being developed and/or are in use. For example, the vaccines based on the Vi polysaccharide are immunogenic and induce protective serum responses. However, these vaccines include a single antigen that is not essential for the virulence of the bacterium (Vi negative variants of *S.* Typhi are capable of causing disease (141)) and is down-regulated within the tissues soon after infection (142). Multi-antigen vaccines would probably be a wiser choice than single-antigen ones and would also offer the possibility to induce antibody responses that can potentially protect against a large number of *Salmonella* serovars. For example, low-reactogenic outer membrane vesicle-based vaccines that contain multiple structural antigens are very immunogenic and elicit highly functional isotype-switched antibody responses against a variety of polysaccharide and protein determinants (109, 143).

Conclusions.

The overall scenario that emerges from decades of research in experimental animals and in humans indicates that antibodies are certainly essential for resistance against systemic *Salmonella* infections and can express the highest level of protective functions when operating in conjunction with T-cell mediated immunity. Antigen specificity, isotype profile, FcγR receptor usage and complement activation (Figure 3) are intertwined factors that have great influence on antibody-mediated protection to *Salmonella*.

There is still a large deal of discrepancy between findings and conclusions from different studies over several decades and this makes rational vaccine design very difficult.

To improve current vaccines and design new ones in the future it will be necessary to shed light on the mechanisms that underpin both the development of antibody responses and their effector functions. It will also be necessary to learn how to tailor antibody responses to situations where other components of the immune system might be impaired, as often seen in endemic areas where immune-suppressive co-morbidities geographically coincide with systemic *Salmonella* infections. Different vaccines and antibody responses may be needed for travellers and residents in endemic areas.

Figures

Figure 1

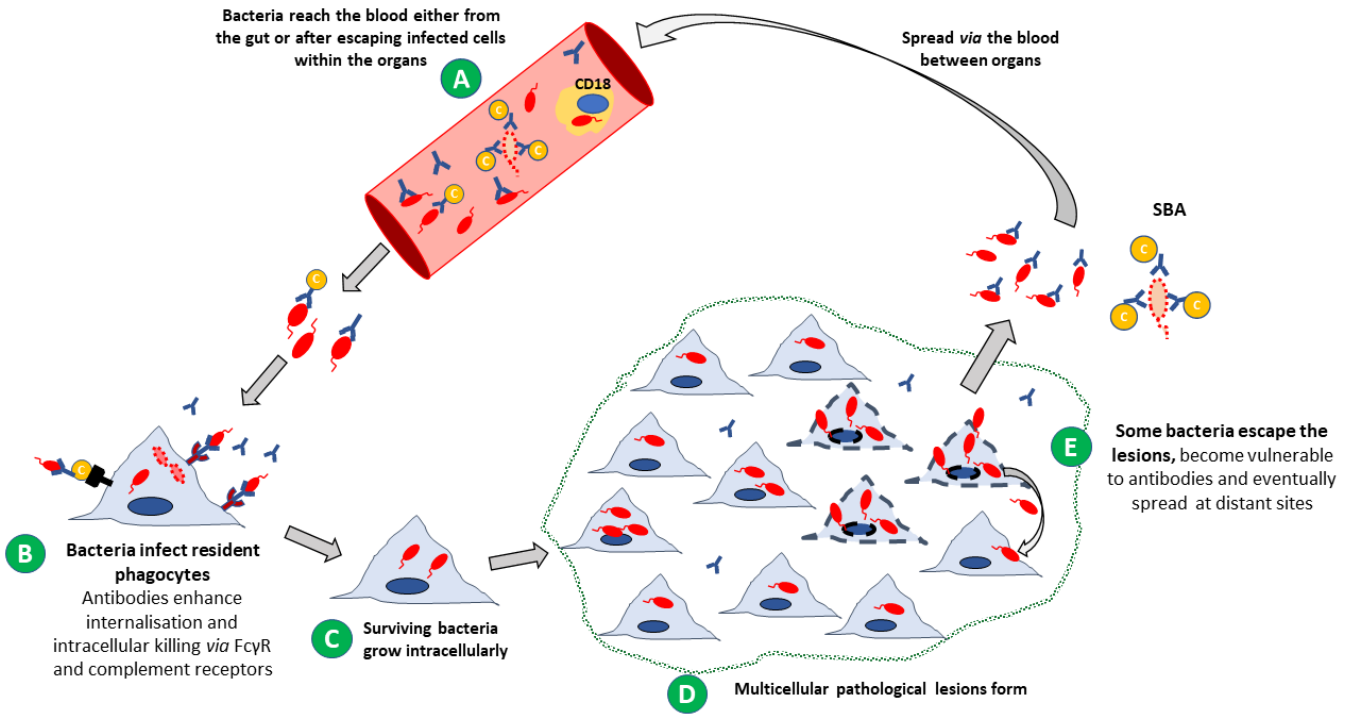
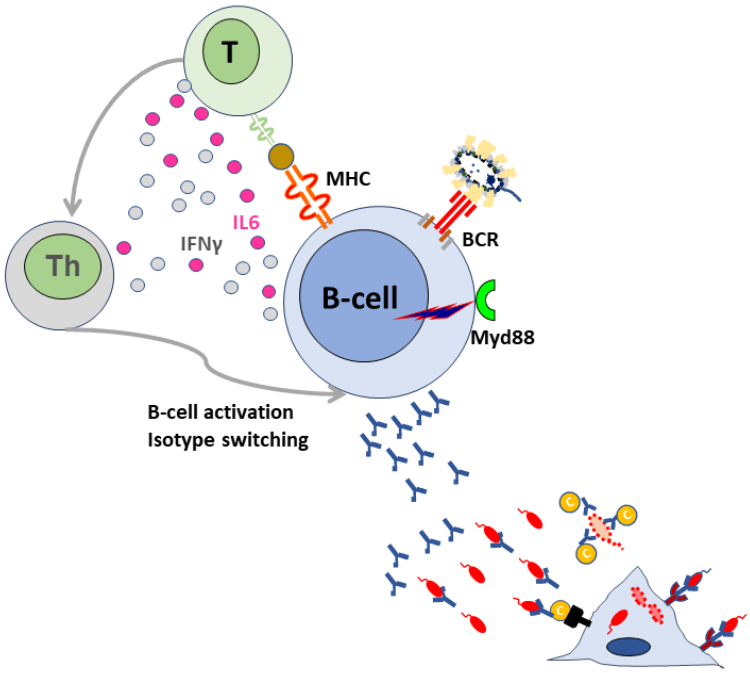


Figure 1. The complex pathogenesis of *Salmonella:* an intracellular antibody-refractive growth phase and an extracellular antibody-susceptible phase of spread. A) After invading the gut, *Salmonella* reach

the blood where can be targeted by antibodies. Some bacteria in the blood are lysed by antibody- and complement-dependent serum bactericidal activity (SBA). **B)** Bacteria opsonised by antibodies are engulfed and killed more efficiently by resident phagocytes as soon as they reach the tissues (*e.g.* spleen, liver, bone marrow). **C)** Those bacteria that resist killing establish unicellular initial infection foci (infected phagocytes). Surviving bacteria grow mainly in an intracellular location. **D)** The initial single-cell infection foci become spatially separated multicellular pathological lesions due to the infiltration of polymorphonuclear phagocytes (PMNs) and later mononuclear cells. In this intracellular location bacteria are inaccessible to antibodies. Bacteria grow intracellularly within the multicellular infection foci, but their numbers within each phagocyte remain low due to the spread of the infection to infection of new host cells. **E)** Some bacteria escape the lesions, become vulnerable to antibodies and eventually spread at distant sites. When the bacteria are released from infected cells, they might undergo three different fates: I) be targeted by antibodies, and killed *via* complement-mediated SBA or opsonophagocytos; II) rapidly infect neighbouring cells within the same lesion; III) travel to distant sites in the body to establish new unicellular infection foci.

Figure 2



406

407 **Figure 2. B-cells and T-cells crosstalk impact on quality of the antibody response.** MyD88 signalling and
408 recognition of bacterial antigens *via* B-cell Receptor (BcR) leads to B-cell cell activation and antigen
409 presentation to naïve T-cells *via* MHC Class II molecules. IL6 and IFNγ from B-cells mediate the
410 development of Th immunity. Th immunity in turn triggers activation and maturation of B-cells and induce
411 isotype switching of the antibody response.

Figure 3

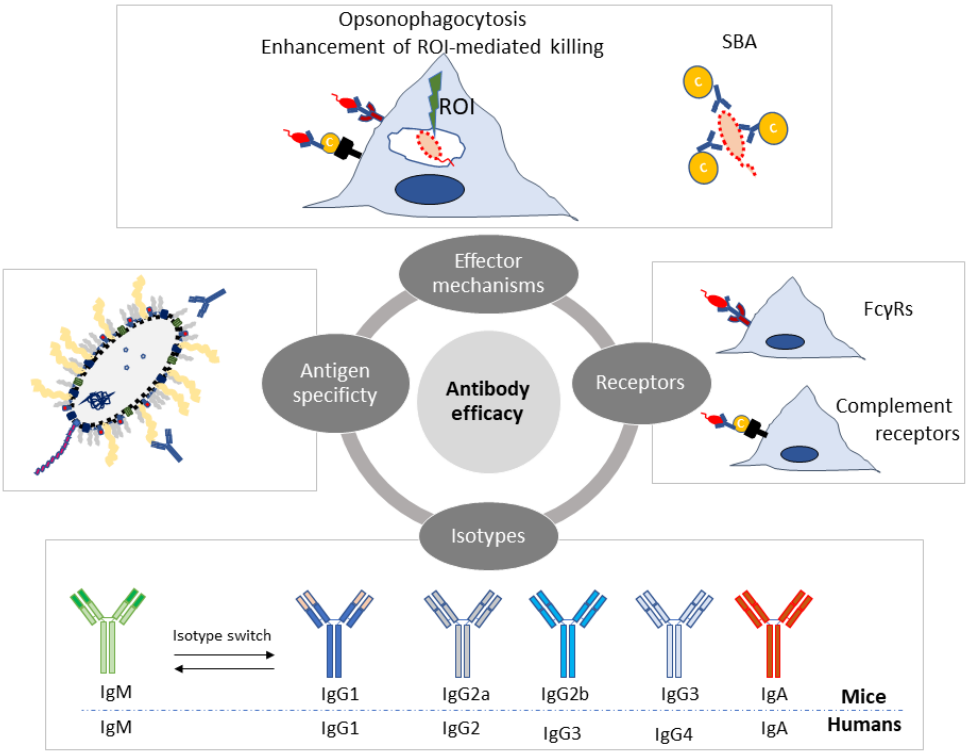


Figure 3. Antibody-mediated protection to *Salmonella* is underpinned by a complex interplay between qualitative traits and effector mechanisms. Antigen specificity, isotype profile, FcγR receptor usage, complement activation, and different effector mechanisms (opsonophagocytosis, killing *via* reactive oxygen intermediates (ROI) and serum bactericidal activity (SBA)) are intertwined factors that influence antibody-mediated protection to *Salmonella*.

References.

1. Breiman RF, Cosmas L, Njuguna H, Audi A, Olack B, Ochieng JB, Wamola N, Bigogo GM, Awiti G, Tabu CW, Burke H, Williamson J, Oundo JO, Mintz ED, Feikin DR. 2012. Population-based incidence of typhoid fever in an urban informal settlement and a rural area in Kenya: implications for typhoid vaccine use in Africa. PLoS One 7:e29119.

2. Crump JA, Luby SP, Mintz ED. 2004. The global burden of typhoid fever. Bull World Health Organ 82:346-53.
3. Crump JA, Mintz ED. 2010. Global trends in typhoid and paratyphoid Fever. Clin Infect Dis 50:241-6.
4. Lutterloh E, Likaka A, Sejvar J, Manda R, Naiene J, Monroe SS, Khaila T, Chilima B, Mallewa M, Kampondeni SD, Lowther SA, Capewell L, Date K, Townes D, Redwood Y, Schier JG, Nygren B, Tippett Barr B, Demby A, Phiri A, Lungu R, Kaphiyo J, Humphrys M, Talkington D, Joyce K, Stockman LJ, Armstrong GL, Mintz E. 2012. Multidrug-resistant typhoid fever with neurologic findings on the Malawi-Mozambique border. Clin Infect Dis 54:1100-6.
5. Neil KP, Sodha SV, Lukwago L, S OT, Mikoleit M, Simington SD, Mukobi P, Balinandi S, Majalija S, Ayers J, Kagirita A, Wefula E, Asiimwe F, Kweyamba V, Talkington D, Shieh WJ, Adem P, Batten BC, Zaki SR, Mintz E. 2012. A large outbreak of typhoid fever associated with a high rate of intestinal perforation in Kasese District, Uganda, 2008-2009. Clin Infect Dis 54:1091-9.
6. Crump JA, Heyderman RS. 2014. Invasive Salmonella infections in Africa. Trans R Soc Trop Med Hyg 108:673-5.
7. Crump JA, Sjolund-Karlsson M, Gordon MA, Parry CM. 2015. Epidemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance, and Antimicrobial Management of Invasive Salmonella Infections. Clin Microbiol Rev 28:901-37.
8. Feasey NA, Dougan G, Kingsley RA, Heyderman RS, Gordon MA. 2012. Invasive non-typhoidal salmonella disease: an emerging and neglected tropical disease in Africa. Lancet 379:2489-99.
9. Gordon MA. 2008. Salmonella infections in immunocompromised adults. J Infect 56:413-22.

10. Gordon MA, Graham SM, Walsh AL, Wilson L, Phiri A, Molyneux E, Zijlstra EE, Heyderman RS, Hart CA, Molyneux ME. 2008. Epidemics of invasive Salmonella enterica serovar enteritidis and S. enterica Serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi. Clin Infect Dis 46:963-9.

11. Kariuki S, Gordon MA, Feasey N, Parry CM. 2015. Antimicrobial resistance and management of invasive Salmonella disease. Vaccine 33 Suppl 3:C21-C29.

12. Reddy EA, Shaw AV, Crump JA. 2010. Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. Lancet Infect Dis 10:417-32.

13. Cooke FJ, Wain J. 2004. The emergence of antibiotic resistance in typhoid fever. Travel Med Infect Dis 2:67-74.

14. Harish BN, Menezes GA. 2011. Antimicrobial resistance in typhoidal salmonellae. Indian J Med Microbiol 29:223-9.

15. Klemm EJ, Shakoor S, Page AJ, Qamar FN, Judge K, Saeed DK, Wong VK, Dallman TJ, Nair S, Baker S, Shaheen G, Qureshi S, Yousafzai MT, Saleem MK, Hasan Z, Dougan G, Hasan R. 2018. Emergence of an Extensively Drug-Resistant Salmonella enterica Serovar Typhi Clone Harboring a Promiscuous Plasmid Encoding Resistance to Fluoroquinolones and Third-Generation Cephalosporins. MBio 9.

16. Baker SJ, Payne DJ, Rappuoli R, De Gregorio E. 2018. Technologies to address antimicrobial resistance. Proc Natl Acad Sci U S A 115:12887-12895.

17. Bloom DE, Black S, Salisbury D, Rappuoli R. 2018. Antimicrobial resistance and the role of vaccines. Proc Natl Acad Sci U S A 115:12868-12871.

18. Klugman KP, Black S. 2018. Impact of existing vaccines in reducing antibiotic resistance: Primary and secondary effects. Proc Natl Acad Sci U S A 115:12896-12901.

19. Tagliabue A, Leite LCC, Leroy OY, Rappuoli R. 2019. Editorial: A Global Perspective on Vaccines: Priorities, Challenges and Online Information. Front Immunol 10:2556.

20. MacLennan CA, Martin LB, Micoli F. 2014. Vaccines against invasive Salmonella disease: current status and future directions. Hum Vaccin Immunother 10:1478-93.

21. Mastroeni P, Chabalgoity JA, Dunstan SJ, Maskell DJ, Dougan G. 2001. Salmonella: immune responses and vaccines. Vet J 161:132-64.

22. Harrison JA, Villarreal-Ramos B, Mastroeni P, Demarco de Hormaeche R, Hormaeche CE. 1997. Correlates of protection induced by live Aro- Salmonella typhimurium vaccines in the murine typhoid model. Immunology 90:618-25.

23. Kotlarski I, Pope M, Doherty K, Attridge SR. 1989. The in vitro proliferative response of lymphoid cells of mice infected with Salmonella enteritidis 11RX. Immunol Cell Biol 67 (Pt 1):19-29.

24. Grant AJ, Oshota O, Chaudhuri RR, Mayho M, Peters SE, Clare S, Maskell DJ, Mastroeni P. 2016. Genes Required for the fitness of Salmonella enterica serovar Typhimurium During Infection of Immunodeficient gp91-/-phox Mice. Infect Immun doi:10.1128/IAI.01423-15.

25. Hess J, Ladel C, Miko D, Kaufmann SH. 1996. Salmonella typhimurium aroA- infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. J Immunol 156:3321-6.

26. Muotiala A. 1992. Anti-IFN-gamma-treated mice--a model for testing safety of live Salmonella vaccines. Vaccine 10:243-6.

27. Sinha K, Mastroeni P, Harrison J, de Hormaeche RD, Hormaeche CE. 1997. Salmonella typhimurium aroA, htrA, and aroD htrA mutants cause progressive infections in athymic (nu/nu) BALB/c mice. Infect Immun 65:1566-9.

28. Lokken KL, Mooney JP, Butler BP, Xavier MN, Chau JY, Schaltenberg N, Begum RH, Muller W, Luckhart S, Tsolis RM. 2014. Malaria Parasite Infection Compromises Control of Concurrent Systemic Non-typhoidal Salmonella Infection via IL-10-Mediated Alteration of Myeloid Cell Function. PLoS Pathog 10:e1004049.

29. Roux CM, Butler BP, Chau JY, Paixao TA, Cheung KW, Santos RL, Luckhart S, Tsolis RM. 2010. Both hemolytic anemia and malaria parasite-specific factors increase susceptibility to Nontyphoidal Salmonella enterica serovar typhimurium infection in mice. Infect Immun 78:1520-7.

30. Feasey NA, Everett D, Faragher EB, Roca-Feltrer A, Kang'ombe A, Denis B, Kerac M, Molyneux E, Molyneux M, Jahn A, Gordon MA, Heyderman RS. 2015. Modelling the Contributions of Malaria, HIV, Malnutrition and Rainfall to the Decline in Paediatric Invasive Non-typhoidal Salmonella Disease in Malawi. PLoS Negl Trop Dis 9:e0003979.

31. Gordon MA. 2011. Invasive nontyphoidal Salmonella disease: epidemiology, pathogenesis and diagnosis. Curr Opin Infect Dis 24:484-9.

32. Scott JA, Berkley JA, Mwangi I, Ochola L, Uyoga S, Macharia A, Ndila C, Lowe BS, Mwarumba S, Bauni E, Marsh K, Williams TN. 2011. Relation between falciparum malaria and bacteraemia in Kenyan children: a population-based, case-control study and a longitudinal study. Lancet 378:1316-23.

33. Garvis SG, Beuzon CR, Holden DW. 2001. A role for the PhoP/Q regulon in inhibition of fusion between lysosomes and Salmonella-containing vacuoles in macrophages. Cell Microbiol 3:731-44.

34. Helaine S, Thompson JA, Watson KG, Liu M, Boyle C, Holden DW. 2010. Dynamics of intracellular bacterial replication at the single cell level. Proc Natl Acad Sci U S A 107:3746-51.

35. Shea JE, Beuzon CR, Gleeson C, Mundy R, Holden DW. 1999. Influence of the Salmonella typhimurium pathogenicity island 2 type III secretion system on bacterial growth in the mouse. Infect Immun 67:213-9.

36. Richter-Dahlfors A, Buchan AMJ, Finlay BB. 1997. Murine salmonellosis studied by confocal microscopy: Salmonella typhimurium resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. J Exp Med 186:569-80.

37. Vazquez-Torres A, Xu Y, Jones-Carson J, Holden DW, Lucia SM, Dinauer MC, Mastroeni P, Fang FC. 2000. Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science 287:1655-8.

38. Fields PI, Swanson RV, Haidaris CG, Heffron F. 1986. Mutants of Salmonella typhimurium that cannot survive within the macrophage are avirulent. Proc Natl Acad Sci U S A 83:5189-93.

39. Gordon MA, Gordon SB, Musaya L, Zijlstra EE, Molyneux ME, Read RC. 2007. Primary macrophages from HIV-infected adults show dysregulated cytokine responses to Salmonella, but normal internalization and killing. AIDS 21:2399-408.

40. Gordon MA, Kankwatira AM, Mwafulirwa G, Walsh AL, Hopkins MJ, Parry CM, Faragher EB, Zijlstra EE, Heyderman RS, Molyneux ME. 2010. Invasive non-typhoid salmonellae establish

systemic intracellular infection in HIV-infected adults: an emerging disease pathogenesis. Clin Infect Dis 50:953-62.

41. Harrington KA, Hormaeche CE. 1986. Expression of the innate resistance gene Ity in mouse Kupffer cells infected with Salmonella typhimurium in vitro. Microb Pathog 1:269-74.

42. Hormaeche CE. 1979. The natural resistance of radiation chimeras to S. typhimurium C5. Immunology 37:329-32.

43. Hormaeche CE. 1979. Genetics of natural resistance to salmonellae in mice. Immunology 37:319-27.

44. Hormaeche CE. 1979. Natural resistance to Salmonella typhimurium in different inbred mouse strains. Immunology 37:311-8.

45. Hsu HS. 1989. Pathogenesis and immunity in murine salmonellosis. Microbiol Rev 53:390-409.

46. Collins FM. 1974. Vaccines and cell-mediated immunity. Bacteriol Rev 38:371-402.

47. Mackaness GB, Blanden RV, Collins FM. 1966. Host-parasite relations in mouse typhoid. J Exp Med 124:573-83.

48. Acharya IL, Lowe CU, Thapa R, Gurubacharya VL, Shrestha MB, Cadoz M, Schulz D, Armand J, Bryla DA, Trollfors B, et al. 1987. Prevention of typhoid fever in Nepal with the Vi capsular polysaccharide of Salmonella typhi. A preliminary report. N Engl J Med 317:1101-4.

49. Levine MM, Ferreccio C, Black RE, Tacket CO, Germanier R. 1989. Progress in vaccines against typhoid fever. Rev Infect Dis 11 Suppl 3:S552-67.

50. Eisenstein TK, Killar LM, Sultzer BM. 1984. Immunity to infection with Salmonella typhimurium: mouse-strain differences in vaccine- and serum-mediated protection. J Infect Dis 150:425-35.

549 51. Szu SC, Stone AL, Robbins JD, Schneerson R, Robbins JB. 1987. Vi capsular polysaccharide-protein
550 conjugates for prevention of typhoid fever. Preparation, characterization, and immunogenicity in
551 laboratory animals. J Exp Med 166:1510-24.
552 52. Thiem VD, Lin FY, Canh DG, Son NH, Anh DD, Mao ND, Chu C, Hunt SW, Robbins JB, Schneerson
553 R, Szu SC. 2011. The Vi conjugate typhoid vaccine is safe, elicits protective levels of IgG anti-Vi,
554 and is compatible with routine infant vaccines. Clin Vaccine Immunol 18:730-5.
555 53. Szu SC. 2013. Development of Vi conjugate - a new generation of typhoid vaccine. Expert Rev
556 Vaccines 12:1273-86.
557 54. Mastroeni P, Villarreal-Ramos B, Hormaeche CE. 1993. Adoptive transfer of immunity to oral
558 challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune
559 serum and T cells. Infect Immun 61:3981-4.
560 55. Gondwe EN, Molyneux ME, Goodall M, Graham SM, Mastroeni P, Drayson MT, MacLennan CA.
561 2010. Importance of antibody and complement for oxidative burst and killing of invasive
562 nontyphoidal Salmonella by blood cells in Africans. Proc Natl Acad Sci U S A 107:3070-5.
563 56. MacLennan CA, Gondwe EN, Msefula CL, Kingsley RA, Thomson NR, White SA, Goodall M, Pickard
564 DJ, Graham SM, Dougan G, Hart CA, Molyneux ME, Drayson MT. 2008. The neglected role of
565 antibody in protection against bacteremia caused by nontyphoidal strains of Salmonella in
566 African children. J Clin Invest 118:1553-62.
567 57. Nyirenda TS, Gilchrist JJ, Feasey NA, Glennie SJ, Bar-Zeev N, Gordon MA, MacLennan CA,
568 Mandala WL, Heyderman RS. 2014. Sequential acquisition of T cells and antibodies to
569 nontyphoidal Salmonella in Malawian children. J Infect Dis 210:56-64.

570 58. Cunnington AJ, de Souza JB, Walther M, Riley EM. 2012. Malaria impairs resistance to Salmonella
571 through heme- and heme oxygenase-dependent dysfunctional granulocyte mobilization. Nat
572 Med 18:120-7.
573 59. Cunnington AJ, Riley EM. 2010. Suppression of vaccine responses by malaria: insignificant or
574 overlooked? Expert Rev Vaccines 9:409-29.
575 60. Gordon MA, Banda HT, Gondwe M, Gordon SB, Boeree MJ, Walsh AL, Corkill JE, Hart CA, Gilks CF,
576 Molyneux ME. 2002. Non-typhoidal salmonella bacteraemia among HIV-infected Malawian
577 adults: high mortality and frequent recrudescence. Aids 16:1633-41.
578 61. Greenwood BM, Brueton MJ. 1974. Complement activation in children with acute malaria. Clin
579 Exp Immunol 18:267-72.
580 62. Mooney JP, Lee SJ, Lokken KL, Nanton MR, Nuccio SP, McSorley SJ, Tsolis RM. 2015. Transient
581 Loss of Protection Afforded by a Live Attenuated Non-typhoidal Salmonella Vaccine in Mice Co-
582 infected with Malaria. PLoS Negl Trop Dis 9:e0004027.
583 63. Klemm EJ, Gkrania-Klotsas E, Hadfield J, Forbester JL, Harris SR, Hale C, Heath JN, Wileman T,
584 Clare S, Kane L, Goulding D, Otto TD, Kay S, Doffinger R, Cooke FJ, Carmichael A, Lever AM,
585 Parkhill J, MacLennan CA, Kumararatne D, Dougan G, Kingsley RA. 2016. Emergence of host-
586 adapted Salmonella Enteritidis through rapid evolution in an immunocompromised host. Nat
587 Microbiol 1:15023.
588 64. de Jong R, Altare F, Haagen IA, Elferink DG, Boer T, van Breda Vriesman PJ, Kabel PJ, Draaisma
589 JM, van Dissel JT, Kroon FP, Casanova JL, Ottenhoff TH. 1998. Severe mycobacterial and
590 Salmonella infections in interleukin-12 receptor-deficient patients. Science 280:1435-8.

65. Jouanguy E, Doffinger R, Dupuis S, Pallier A, Altare F, Casanova JL. 1999. IL-12 and IFN-gamma in host defense against mycobacteria and salmonella in mice and men. Curr Opin Immunol 11:346-51.

66. MacLennan C, Fieschi C, Lammas DA, Picard C, Dorman SE, Sanal O, MacLennan JM, Holland SM, Ottenhoff TH, Casanova JL, Kumararatne DS. 2004. Interleukin (IL)-12 and IL-23 are key cytokines for immunity against Salmonella in humans. J Infect Dis 190:1755-7.

67. Picard C, Fieschi C, Altare F, Al-Jumaah S, Al-Hajjar S, Feinberg J, Dupuis S, Soudais C, Al-Mohsen IZ, Genin E, Lammas D, Kumararatne DS, Leclerc T, Rafii A, Frayha H, Murugasu B, Wah LB, Sinniah R, Loubser M, Okamoto E, Al-Ghonaium A, Tufenkeji H, Abel L, Casanova JL. 2002. Inherited interleukin-12 deficiency: IL12B genotype and clinical phenotype of 13 patients from six kindreds. Am J Hum Genet 70:336-48.

68. Carter PB, Collins FM. 1974. The route of enteric infection in normal mice. J Exp Med 139:1189-203.

69. Vazquez-Torres A, Jones-Carson J, Baumler AJ, Falkow S, Valdivia R, Brown W, Le M, Berggren R, Parks WT, Fang FC. 1999. Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. Nature 401:804-8.

70. Biozzi G, Howard JG, Halpern BN, Stiffel C, Mouton D. 1960. The kinetics of blood clearance of isotopically labelled Salmonella enteritidis by the reticuloendothelial system in mice. Immunology 3:74-89.

71. Coward C, Restif O, Dybowski R, Grant AJ, Maskell DJ, Mastroeni P. 2014. The effects of vaccination and immunity on bacterial infection dynamics in vivo. PLoS Pathog 10:e1004359.

612 72. Warren J, Mastroeni P, Dougan G, Noursadeghi M, Cohen J, Walport MJ, Botto M. 2002.
613 Increased susceptibility of C1q-deficient mice to Salmonella enterica serovar Typhimurium
614 infection. Infect Immun 70:551-7.
615 73. Dunlap NE, Benjamin WH, Jr., McCall RD, Jr., Tilden AB, Briles DE. 1991. A 'safe-site' for
616 Salmonella typhimurium is within splenic cells during the early phase of infection in mice. Microb
617 Pathog 10:297-310.
618 74. Salcedo SP, Noursadeghi M, Cohen J, Holden DW. 2001. Intracellular replication of Salmonella
619 typhimurium strains in specific subsets of splenic macrophages in vivo. Cell Microbiol 3:587-97.
620 75. Dunlap NE, Benjamin WH, Jr., Berry AK, Eldridge JH, Briles DE. 1992. A 'safe-site' for Salmonella
621 typhimurium is within splenic polymorphonuclear cells. Microb Pathog 13:181-90.
622 76. Yrlid U, Svensson M, Hakansson A, Chambers BJ, Ljunggren HG, Wick MJ. 2001. In vivo activation
623 of dendritic cells and T cells during Salmonella enterica serovar Typhimurium infection. Infect
624 Immun 69:5726-35.
625 77. Sheppard M, Webb C, Heath F, Mallows V, Emilianus R, Maskell D, Mastroeni P. 2003. Dynamics
626 of bacterial growth and distribution within the liver during Salmonella infection. Cell Microbiol
627 5:593-600.
628 78. Mastroeni P, Skepper JN, Hormaeche CE. 1995. Effect of anti-tumor necrosis factor alpha
629 antibodies on histopathology of primary Salmonella infections [published erratum appears in
630 Infect Immun 1995 Dec;63(12):4966]. Infect Immun 63:3674-82.
631 79. Shea JE, Hensel M, Gleeson C, Holden DW. 1996. Identification of a virulence locus encoding a
632 second type III secretion system in Salmonella typhimurium. Proc Natl Acad Sci U S A 93:2593-7.

80. Brown SP, Cornell SJ, Sheppard M, Grant AJ, Maskell DJ, Grenfell BT, Mastroeni P. 2006. Intracellular demography and the dynamics of Salmonella enterica infections. PLoS Biol 4:e349.
81. Grant AJ, Morgan FJ, McKinley TJ, Foster GL, Maskell DJ, Mastroeni P. 2012. Attenuated Salmonella Typhimurium lacking the pathogenicity island-2 type 3 secretion system grow to high bacterial numbers inside phagocytes in mice. PLoS Pathog 8:e1003070.
82. Mastroeni P, Arena A, Costa GB, Liberto MC, Bonina L, Hormaeche CE. 1991. Serum TNF alpha in mouse typhoid and enhancement of a Salmonella infection by anti-TNF alpha antibodies. Microb Pathog 11:33-8.
83. Mastroeni P, Clare S, Khan S, Harrison JA, Hormaeche CE, Okamura H, Kurimoto M, Dougan G. 1999. Interleukin 18 contributes to host resistance and gamma interferon production in mice infected with virulent Salmonella typhimurium. Infect Immun 67:478-83.
84. Mastroeni P, Harrison JA, Chabalgoity JA, Hormaeche CE. 1996. Effect of interleukin 12 neutralization on host resistance and gamma interferon production in mouse typhoid. Infect Immun 64:189-96.
85. Mastroeni P, Villarreal-Ramos B, Hormaeche CE. 1992. Role of T cells, TNF alpha and IFN gamma in recall of immunity to oral challenge with virulent salmonellae in mice vaccinated with live attenuated aro- Salmonella vaccines. Microb Pathog 13:477-91.
86. Muotiala A, Makela PH. 1990. The role of IFN-gamma in murine Salmonella typhimurium infection. Microb Pathog 8:135-41.
87. Goh YS, Armour KL, Clark MR, Grant AJ, Mastroeni P. 2016. Igg Subclasses Targeting the Flagella of Salmonella enterica Serovar Typhimurium Can Mediate Phagocytosis and Bacterial Killing. J Vaccines Vaccin 7.

88. Uppington H, Menager N, Boross P, Wood J, Sheppard M, Verbeek S, Mastroeni P. 2006. Effect of immune serum and role of individual Fcgamma receptors on the intracellular distribution and survival of Salmonella enterica serovar Typhimurium in murine macrophages. Immunology 119:147-58.

89. Grant AJ, Restif O, McKinley TJ, Sheppard M, Maskell DJ, Mastroeni P. 2008. Modelling within-host spatiotemporal dynamics of invasive bacterial disease. PLoS Biol 6:e74.

90. Brown A, Hormaeche CE, Demarco-de-Hormaeche R, Winther M, Dougan G, Maskell DJ, Stocker BA. 1987. An attenuated aroA Salmonella typhimurium vaccine elicits humoral and cellular immunity to cloned beta-galactosidase in mice. J Infect Dis 155:86-92.

91. Liang L, Juarez S, Nga TV, Dunstan S, Nakajima-Sasaki R, Davies DH, McSorley S, Baker S, Felgner PL. 2013. Immune profiling with a Salmonella Typhi antigen microarray identifies new diagnostic biomarkers of human typhoid. Sci Rep 3:1043.

92. Villarreal-Ramos B, Manser J, Collins RA, Dougan G, Chatfield SN, Howard CJ. 1998. Immune responses in calves immunised orally or subcutaneously with a live Salmonella typhimurium aro vaccine. Vaccine 16:45-54.

93. Brown A, Hormaeche CE. 1989. The antibody response to salmonellae in mice and humans studied by immunoblots and ELISA. Microb Pathog 6:445-54.

94. Tran TH, Nguyen TD, Nguyen TT, Ninh TT, Tran NB, Nguyen VM, Tran TT, Cao TT, Pham VM, Nguyen TC, Tran TD, Pham VT, To SD, Campbell JI, Stockwell E, Schultsz C, Simmons CP, Glover C, Lam W, Marques F, May JP, Upton A, Budhram R, Dougan G, Farrar J, Nguyen VV, Dolecek C. 2010. A randomised trial evaluating the safety and immunogenicity of the novel single oral dose typhoid vaccine M01ZH09 in healthy Vietnamese children. PLoS One 5:e11778.

677 95. Murphy JR, Baqar S, Munoz C, Schlesinger L, Ferreccio C, Lindberg AA, Svenson S, Losonsky G,
678 Koster F, Levine MM. 1987. Characteristics of humoral and cellular immunity to Salmonella typhi
679 in residents of typhoid-endemic and typhoid-free regions. J Infect Dis 156:1005-9.
680 96. Sztein MB. 2007. Cell-mediated immunity and antibody responses elicited by attenuated
681 Salmonella enterica Serovar Typhi strains used as live oral vaccines in humans. Clin Infect Dis 45
682 Suppl 1:S15-9.
683 97. Hormaeche CE, Mastroeni P, Harrison JA, Demarco de Hormaeche R, Svenson S, Stocker BA.
684 1996. Protection against oral challenge three months after i.v. immunization of BALB/c mice with
685 live Aro Salmonella typhimurium and Salmonella enteritidis vaccines is serotype (species)-
686 dependent and only partially determined by the main LPS O antigen. Vaccine 14:251-9.
687 98. Segall T, Lindberg AA. 1993. Oral vaccination of calves with an aromatic-dependent Salmonella
688 dublin (O9,12) hybrid expressing O4,12 protects against S. dublin (O9,12) but not against
689 Salmonella typhimurium (O4,5,12). Infect Immun 61:1222-31.
690 99. Svenson SB, Lindberg AA. 1981. Artificial Salmonella vaccines: Salmonella typhimurium O-
691 antigen- specific oligosaccharide-protein conjugates elicit protective antibodies in rabbits and
692 mice. Infect Immun 32:490-6.
693 100. Carlin NI, Svenson SB, Lindberg AA. 1987. Role of monoclonal O-antigen antibody epitope
694 specificity and isotype in protection against experimental mouse typhoid. Microb Pathog 2:171-
695 83.
696 101. Hale C, Bowe F, Pickard D, Clare S, Haeuw JF, Powers U, Menager N, Mastroeni P, Dougan G.
697 2006. Evaluation of a novel Vi conjugate vaccine in a murine model of salmonellosis. Vaccine
698 24:4312-20.

102. Jin C, Gibani MM, Moore M, Juel HB, Jones E, Meiring J, Harris V, Gardner J, Nebykova A, Kerridge SA, Hill J, Thomaides-Brears H, Blohmke CJ, Yu LM, Angus B, Pollard AJ. 2017. Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine in the prevention of typhoid fever using a controlled human infection model of Salmonella Typhi: a randomised controlled, phase 2b trial. Lancet 390:2472-2480.

103. Shakya M, Colin-Jones R, Theiss-Nyland K, Voysey M, Pant D, Smith N, Liu X, Tonks S, Mazur O, Farooq YG, Clarke J, Hill J, Adhikari A, Dongol S, Karkey A, Bajracharya B, Kelly S, Gurung M, Baker S, Neuzil KM, Shrestha S, Basnyat B, Pollard AJ, Ty VACNST. 2019. Phase 3 Efficacy Analysis of a Typhoid Conjugate Vaccine Trial in Nepal. N Engl J Med 381:2209-2218.

104. Kuusi N, Nurminen M, Saxen H, Valtonen M, Makela PH. 1979. Immunization with major outer membrane proteins in experimental salmonellosis of mice. Infect Immun 25:857-62.

105. Dominguez-Medina CC, Perez-Toledo M, Schager AE, Marshall JL, Cook CN, Bobat S, Hwang H, Chun BJ, Logan E, Bryant JA, Channell WM, Morris FC, Jossi SE, Alshayea A, Rossiter AE, Barrow PA, Horsnell WG, MacLennan CA, Henderson IR, Lakey JH, Gumbart JC, Lopez-Macias C, Bavro VN, Cunningham AF. 2020. Outer membrane protein size and LPS O-antigen define protective antibody targeting to the Salmonella surface. Nat Commun 11:851.

106. Gil-Cruz C, Bobat S, Marshall JL, Kingsley RA, Ross EA, Henderson IR, Leyton DL, Coughlan RE, Khan M, Jensen KT, Buckley CD, Dougan G, MacLennan IC, Lopez-Macias C, Cunningham AF. 2009. The porin OmpD from nontyphoidal Salmonella is a key target for a protective B1b cell antibody response. Proc Natl Acad Sci U S A 106:9803-8.

719 107. Goh YS, Grant AJ, Restif O, McKinley TJ, Armour KL, Clark MR, Mastroeni P. 2011. Human IgG
720 isotypes and activating Fcgamma receptors in the interaction of Salmonella enterica serovar
721 Typhimurium with phagocytic cells. Immunology 133:74-83.
722 108. Wahid R, Zafar SJ, McArthur MA, Pasetti MF, Levine MM, Sztein MB. 2014. Live oral Salmonella
723 enterica serovar Typhi vaccines Ty21a and CVD 909 induce opsonophagocytic functional
724 antibodies in humans that cross-react with S. Paratyphi A and S. Paratyphi B. Clin Vaccine
725 Immunol 21:427-34.
726 109. Micoli F, Rondini S, Alfini R, Lanzilao L, Necchi F, Negrea A, Rossi O, Brandt C, Clare S, Mastroeni
727 P, Rappuoli R, Saul A, MacLennan CA. 2018. Comparative immunogenicity and efficacy of
728 equivalent outer membrane vesicle and glycoconjugate vaccines against nontyphoidal
729 Salmonella. Proc Natl Acad Sci U S A 115:10428-10433.
730 110. Siggins MK, O'Shaughnessy CM, Pravin J, Cunningham AF, Henderson IR, Drayson MT,
731 MacLennan CA. 2014. Differential timing of antibody-mediated phagocytosis and cell-free killing
732 of invasive African Salmonella allows immune evasion. Eur J Immunol 44:1093-8.
733 111. Juel HB, Thomaides-Brears HB, Darton TC, Jones C, Jones E, Shrestha S, Sie R, Eustace A, Galal U,
734 Kurupati P, Van TT, Thieu NTV, Baker S, Blohmke CJ, Pollard AJ. 2017. Salmonella Typhi
735 Bactericidal Antibodies Reduce Disease Severity but Do Not Protect against Typhoid Fever in a
736 Controlled Human Infection Model. Front Immunol 8:1916.
737 112. Rossi O, Coward C, Goh YS, Claassens JWC, MacLennan CA, Verbeek SJ, Mastroeni P. 2019. The
738 essential role of complement in antibody-mediated resistance to Salmonella. Immunology
739 156:69-73.

740 113. Menager N, Foster G, Ugrinovic S, Uppington H, Verbeek S, Mastroeni P. 2007. Fcgamma
741 receptors are crucial for the expression of acquired resistance to virulent Salmonella enterica
742 serovar Typhimurium in vivo but are not required for the induction of humoral or T-cell-
743 mediated immunity. Immunology 120:424-32.
744 114. Lindberg AA, Segall T, Weintraub A, Stocker BA. 1993. Antibody response and protection against
745 challenge in mice vaccinated intraperitoneally with a live aroA O4-O9 hybrid Salmonella dublin
746 strain. Infect Immun 61:1211-21.
747 115. Metcalf ES, O'Brien AD. 1981. Characterization of murine antibody response to Salmonella
748 typhimurium by a class-specific solid-phase radioimmunoassay. Infect Immun 31:33-41.
749 116. Forrest BD, LaBrooy JT, Beyer L, Dearlove CE, Shearman DJ. 1991. The human humoral immune
750 response to Salmonella typhi Ty21a. J Infect Dis 163:336-45.
751 117. Tagliabue A, Villa L, De Magistris MT, Romano M, Silvestri S, Boraschi D, Nencioni L. 1986. IgA-
752 driven T cell-mediated anti-bacterial immunity in man after live oral Ty 21a vaccine. J Immunol
753 137:1504-10.
754 118. Cunningham AF, Gaspal F, Serre K, Mohr E, Henderson IR, Scott-Tucker A, Kenny SM, Khan M,
755 Toellner KM, Lane PJ, MacLennan IC. 2007. Salmonella induces a switched antibody response
756 without germinal centers that impedes the extracellular spread of infection. J Immunol
757 178:6200-7.
758 119. Szu SC, Klugman KP, Hunt S. 2014. Re-examination of immune response and estimation of anti-Vi
759 IgG protective threshold against typhoid fever-based on the efficacy trial of Vi conjugate in
760 young children. Vaccine 32:2359-63.

120. Dahora LC, Jin C, Spreng RL, Feely F, Mathura R, Seaton KE, Zhang L, Hill J, Jones E, Alam SM, Dennison SM, Pollard AJ, Tomaras GD. 2019. IgA and IgG1 Specific to Vi Polysaccharide of Salmonella Typhi Correlate With Protection Status in a Typhoid Fever Controlled Human Infection Model. Front Immunol 10:2582.

121. Saxen H, Makela O. 1982. The protective capacity of immune sera in experimental mouse salmonellosis is mainly due to IgM antibodies. Immunol Lett 5:267-72.

122. Saxen H, Makela O, Svenson SB. 1984. Isotype of protective anti-Salmonella antibodies in experimental mouse salmonellosis. Infect Immun 44:633-6.

123. Goh YS, Clare S, Micoli F, Saul A, Mastroeni P, MacLennan CA. 2015. Monoclonal Antibodies of a Diverse Isotype Induced by an O-Antigen Glycoconjugate Vaccine Mediate In Vitro and In Vivo Killing of African Invasive Nontyphoidal Salmonella. Infect Immun 83:3722-31.

124. Wijburg OL, Uren TK, Simpfendorfer K, Johansen FE, Brandtzaeg P, Strugnell RA. 2006. Innate secretory antibodies protect against natural Salmonella typhimurium infection. J Exp Med 203:21-6.

125. Michetti P, Mahan MJ, Slauch JM, Mekalanos JJ, Neutra MR. 1992. Monoclonal secretory immunoglobulin A protects mice against oral challenge with the invasive pathogen Salmonella typhimurium. Infect Immun 60:1786-92.

126. Zhang Y, Dominguez-Medina C, Cumley NJ, Heath JN, Essex SJ, Bobat S, Schager A, Goodall M, Kracker S, Buckley CD, May RC, Kingsley RA, MacLennan CA, Lopez-Macias C, Cunningham AF, Toellner KM. 2017. IgG1 Is Required for Optimal Protection after Immunization with the Purified Porin OmpD from Salmonella Typhimurium. J Immunol 199:4103-4109.

127. Goh YS, Necchi F, O'Shaughnessy CM, Micoli F, Gavini M, Young SP, Msefula CL, Gondwe EN, Mandala WL, Gordon MA, Saul AJ, MacLennan CA. 2016. Bactericidal Immunity to Salmonella in Africans and Mechanisms Causing Its Failure in HIV Infection. PLoS Negl Trop Dis 10:e0004604.
128. Salerno-Goncalves R, Pasetti MF, Sztein MB. 2002. Characterization of CD8(+) effector T cell responses in volunteers immunized with Salmonella enterica serovar Typhi strain Ty21a typhoid vaccine. J Immunol 169:2196-203.
129. Salerno-Goncalves R, Wyant TL, Pasetti MF, Fernandez-Vina M, Tacket CO, Levine MM, Sztein MB. 2003. Concomitant Induction of CD4(+) and CD8(+) T Cell Responses in Volunteers Immunized with Salmonella enterica Serovar Typhi Strain CVD 908-htrA. J Immunol 170:2734-41.
130. Villarreal B, Mastroeni P, de Hormaeche RD, Hormaeche CE. 1992. Proliferative and T-cell specific interleukin (IL-2/IL-4) production responses in spleen cells from mice vaccinated with aroA live attenuated Salmonella vaccines. Microb Pathog 13:305-15.
131. Mastroeni P, Simmons C, Fowler R, Hormaeche CE, Dougan G. 2000. Igh-6(-/-) (B-cell-deficient) mice fail To mount solid acquired resistance to oral challenge with virulent salmonella enterica serovar typhimurium and show impaired Th1 T-cell responses to salmonella antigens [In Process Citation]. Infect Immun 68:46-53.
132. Ugrinovic S, Menager N, Goh N, Mastroeni P. 2003. Characterization and Development of T-Cell Immune Responses in B-Cell-Deficient (Igh-6(-/-)) Mice with Salmonella enterica Serovar TyphimuriumInfection. Infect Immun 71:6808-19.
133. Mittrucker HW, Raupach B, Kohler A, Kaufmann SH. 2000. Cutting edge: role of B lymphocytes in protective immunity against salmonella typhimurium infection [In Process Citation]. J Immunol 164:1648-52.

134. Nanton MR, Way SS, Shlomchik MJ, McSorley SJ. 2012. Cutting edge: B cells are essential for protective immunity against Salmonella independent of antibody secretion. J Immunol 189:5503-7.

135. Barr TA, Brown S, Mastroeni P, Gray D. 2010. TLR and B cell receptor signals to B cells differentially program primary and memory Th1 responses to Salmonella enterica. J Immunol 185:2783-9.

136. Barr TA, Brown S, Mastroeni P, Gray D. 2009. B cell intrinsic MyD88 signals drive IFN-gamma production from T cells and control switching to IgG2c. J Immunol 183:1005-12.

137. MacLennan CA, Gilchrist JJ, Gordon MA, Cunningham AF, Cobbold M, Goodall M, Kingsley RA, van Oosterhout JJ, Msefula CL, Mandala WL, Leyton DL, Marshall JL, Gondwe EN, Bobat S, Lopez-Macias C, Doffinger R, Henderson IR, Zijlstra EE, Dougan G, Drayson MT, MacLennan IC, Molyneux ME. 2010. Dysregulated humoral immunity to nontyphoidal Salmonella in HIV-infected African adults. Science 328:508-12.

138. Lin FY, Ho VA, Khiem HB, Trach DD, Bay PV, Thanh TC, Kossaczka Z, Bryla DA, Shiloach J, Robbins JB, Schneerson R, Szu SC. 2001. The efficacy of a Salmonella typhi Vi conjugate vaccine in two-to-five-year-old children. N Engl J Med 344:1263-9.

139. Greenwood BM, Bradley-Moore AM, Bryceson AD, Palit A. 1972. Immunosuppression in children with malaria. Lancet 1:169-72.

140. Cunnington AJ, Njie M, Correa S, Takem EN, Riley EM, Walther M. 2012. Prolonged neutrophil dysfunction after Plasmodium falciparum malaria is related to hemolysis and heme oxygenase-1 induction. J Immunol 189:5336-46.

141. Hornick RB, Greisman SE, Woodward TE, DuPont HL, Dawkins AT, Snyder MJ. 1970. Typhoid fever: pathogenesis and immunologic control. N Engl J Med 283:686-91.

142. Janis C, Grant AJ, McKinley TJ, Morgan FJ, John VF, Houghton J, Kingsley RA, Dougan G, Mastroeni P. 2011. In vivo regulation of the Vi antigen in Salmonella and induction of immune responses with an in vivo-inducible promoter. Infect Immun 79:2481-8.

143. Rossi O, Caboni M, Negrea A, Necchi F, Alfini R, Micoli F, Saul A, MacLennan CA, Rondini S, Gerke C. 2016. Toll-Like Receptor Activation by Generalized Modules for Membrane Antigens from Lipid A Mutants of Salmonella enterica Serovars Typhimurium and Enteritidis. Clin Vaccine Immunol 23:304-14.

Author bios.

Pietro Mastroeni obtained a Degree in Medicine and Surgery from the University of Messina, Italy in 1990, a PhD in Immunology from the University and Cambridge in 1994 where he was also awarded the Doctor of Science Degree in 2017. He has worked at the University of Newcastle and Imperial college and is now based at the University of Cambridge. His work has contributed to the fields of microbiology, immunology and vaccine development for the last three decades. He is a Fellow of the Royal Society of Biology and of the American Academy of Microbiology.

Omar Rossi holds a Master of Science in molecular biology and received a PhD in biotechnology at the Novartis Vaccine Institute for Global Health (NVGH) in 2014. After postdoctoral studies at NVGH and being visiting scientist at Wellcome Trust Sanger Institute, he was appointed as research associate at the University of Cambridge, working on pathogenesis of *Salmonella* disease. He is currently a senior scientist in the immunoassay group at GSK Vaccines institute for Global Health, working on development of vaccines, including ones against iNTS, for low and middle income countries. With over ten years of experience in the field of vaccinology, Dr. Rossi's interest is focused on the better understanding of mechanisms underpinning infections and development of vaccines and functional assays to understand the action of antibodies in disease.